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Malaria

Plasmodium vivax and the Duffy antigen: A paradigm revisited

Plasmodium vivax et l'antigène Duffy : un paradigme revisité

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Abstract

The Duffy blood group antigen is the portal of entry of the *Plasmodium vivax* malaria parasite into human red blood cells and the receptor for a number of CXC and CC chemokines. We review here epidemiological data and evidence derived from therapeutic or experimental human infections associating *P. vivax* and the Duffy glycoprotein and laboratory studies indicating that *P. vivax* uses the Duffy antigen as a receptor to invade the red cell. We then review recent field observations indicating that the conclusion of the absolute dependence on the presence of Duffy on the red cell for *P. vivax* infection and development into the red cell no longer holds true and that in some parts of the world, *P. vivax* infects and causes disease in Duffy-negative people.

Keywords: Duffy blood group; *Plasmodium vivax*; Malaria; Receptor; Evolution

Résumé

La glycoprotéine Duffy exposée à la surface du globule rouge est décrite comme le récepteur de *Plasmodium vivax* et de plusieurs chimiokines de type CXC et CC. Dans cette revue, sont résumés les données épidémiologiques et les résultats d'infections thérapeutiques ou expérimentales chez l'homme qui ont permis d'associer *P. vivax* à la protéine Duffy ainsi que les travaux de laboratoire qui démontrent que *P. vivax* utilise l'antigène Duffy comme récepteur pour entrer dans le globule rouge. Sont ensuite discutées, les données récentes de terrain remettant en cause la conclusion que l'interaction entre la protéine Duffy et le parasite est indispensable à l'invasion du globule rouge par *P. vivax* puisque dans certaines régions du monde *P. vivax* peut provoquer des accès palustres chez des sujets Duffy-négatifs.

Mots clés : Groupe sanguin Duffy ; *Plasmodium vivax* ; Paludisme ; Récepteur ; Évolution

1. Introduction

The Duffy blood group antigen is usually described as the portal of entry of the *Plasmodium vivax* malaria parasite into human red blood cells and the promiscuous receptor for a number of CXC and CC chemokines [1,2]. Although there is a growing literature about the role of Duffy on the red cell and on the endothelium as a chemokine receptor, the focus of this paper will be on the relationship of Duffy/DARC with malaria parasites.

The association of *P. vivax* with the Duffy glycoprotein is based on epidemiological evidence and on a large body of infections in humans, either during the decades of malariatherapy in the 20th century when malaria infection was used to treat neurosyphylitic patients or evidence obtained by conducting controlled experimental infection in humans. We will then summarize laboratory studies showing that invasion of *P. vivax* into the red cell requires the Duffy antigen. We will put in perspective the recent field observations indicating that the conclusion of the absolute dependence on the presence of Duffy on the red cell for *P. vivax* infection and development into the

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red cell no longer holds true and that in some parts of the world, *P. vivax* infects and causes disease in Duffy-negative people. There have been reports of *P. vivax* transmission within Duffy negative populations in Western Kenya and a few cases of infections of *P. vivax* infection of Duffy negative erythrocytes in Kenya and Brazil. A recent study in Madagascar however showed that genetically diverse isolates of *P. vivax* cause asymptomatic and symptomatic *vivax* malaria cases, and that the phenomenon was frequent.

2. Duffy blood group antigen

The Duffy antigen was first identified as an antigen expressed on the surface of human red blood cells, its coding sequence was cloned in 1993 and shown to code for a serpentine protein with seven-transmembrane domains [3,4]. The protein is not coupled to G-proteins or other intracellular signalling effectors. The expression of the FY gene is not restricted to the erythrocyte lineage. It is also expressed – and displayed onto the cell surface – by the endothelial cells lining postcapillary venules throughout the body, splenic sinusoids and cerebellar neurons [4–7].

Two co-dominant alleles, FY*A and FY*B, code for the Fya and Fyb blood group antigens, respectively. The basis of this polymorphism is a G-to-A transition at nucleotide 131, resulting in a single amino acid difference, G44D [8]. The FY*A / FY*B frequency shows marked geographic disparities, the FY*B allele being highly predominant in Africa while the FY*A allele is dominant in Asia. The locus has four major phenotypes: Fy(a+b+), Fy(a+b−), Fy(a−b+) and Fy(a−b−) [4]. The molecular basis of the Fy(a−b−) phenotype is a T-to-C transition in the GATA box of the FY gene promoter at position – 33, which disrupts the binding site for the h-GATA1 erythroid lineage transcription factor and silences the cis allele in erythroid cells without affecting its expression in other tissues. First discovered in persons of African descent associated with the FY*B allele, and called FY*B^{ES}, the same mutation has been detected associated with the FY*A allele in individuals living in a *P. vivax*-endemic region of Papua New Guinea (FY*A^{ES}) by Zimmerman et al. [9,10]. The FY*B^{ES} allele is almost fixed in West and Central Africa and as a consequence, the Fy(a−b−) (null) phenotype is the predominant phenotype among populations of West and Central African descent. It is rare among Caucasian, Amerindian, Indian and Asian populations. The FY*A^{ES} mutation is rare, with an allele frequency of 0.022 [10] or (0.012) [11], and appears so far confined to the Melanesian population [12].

An single nucleotide polymorphism at codon 89, resulting in a R89C, a single amino acid substitution in the first cytoplasmic domain, reduces by 90% the level of protein detected onto the erythrocyte and its ability to bind chemokines [13,14]. The mutation is haplotypically associated with the FY*B allele (Fyb^{weak}). The allele has been described in approximately 3.5% of the population.

There is a gene-dosage effect on the expression levels of the Fy antigen on the red cell. Heterozygous carriers of a Duffy-null allele, have an overall expression of the Duffy antigen on their red cell surface reduced by 50% compared to homozygous carriers with a wild-type promoter FY allele [10,15].

3. *Plasmodium vivax*

P. vivax has long been neglected and even today most reports, including the yearly world malaria report of WHO, focus on *P. falciparum* malaria. It is difficult to obtain estimates of the prevalence of *P. vivax* number of clinical cases in the world. *P. vivax* has a wide geographical distribution, is quite frequent in South America and in Asia, where it is frequently the most abundant malaria species today, but it is absent or rare in West and Central Africa. Estimates vary from 70 million to 300 million *P. vivax* clinical cases every year [16–19]. The number of actual infections should be higher as *P. vivax* has the capacity to hide dormant in the liver in healthy people who have recovered from a primary attack (so called hypnozoite forms). Hypnozoites lead to a large prevalence of asymptomatic cases among semi-immune populations. In the last two decades, the situation has been worsened by the emergence and spread of drug-resistant strains of *P. vivax* in Asia and in South America [20,21]. In a number of areas, *P. falciparum* malaria is decreasing but *vivax* malaria is not, and sometimes is on the increase [22].

P. vivax parasites exist in tropical zones but also in some temperate conditions and ecological situations that do not support development of other human malaria species [17,23]. *P. vivax* has a unique biology, including early appearance of gametocytes and transmission potential at low parasite densities. It invades reticulocytes – a characteristic that has so far hampered the development of a reliable long-term culture system. As a consequence, limited in vitro studies have been done on *P. vivax*, including only a handful of studies exploring invasion of the merozoite- a step where the Duffy blood groups comes into play.

4. *Plasmodium vivax* and disease severity

P. vivax is inappropriately considered a benign infection. There is an increasing number of reports and a growing appreciation that *P. vivax* causes signicant morbidity. Severe and fatal *P. vivax* malaria has been documented in Indonesia, Papua New Guinea, Thailand, India and South America. In the Amazonian region of Brazil, the rate of hospital admissions for *P. vivax* infections has recently increased, while those of *P. falciparum* have decreased [24]; numerous cases of severe, fatal *P. vivax* malaria have been documented (reviewed in [22]). In other regions, from 20 to 40% of hospital admissions for malaria have *P. vivax* mono-infection. In Papua, Indonesia, the overall mortality among those hospitalized with *P. vivax* was no different from that observed with *P. falciparum* and similar case fatality rates were observed in infants for *falciparum* and *vivax* malaria [25–28].

Severe anaemia is a frequent life threatening clinical manifestation. *P. vivax* infections induce a greater inflammation in the lungs than is observed in *P. falciparum* infections [29,30]. Whether *P. vivax* is the causal agent of death or contributes to fatal outcome in patients with other co-morbidity is unclear. Be

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it as it is, this question does not dismiss a role for *P. vivax* in malaria-related mortality that has been overlooked. As such *P. vivax* should not be viewed as a ''benign'' pathogen unlikely to act as a selective agent on the human species.

This being said, there seems to be substantial heterogeneity in the clinical spectrum of the disease which is probably attributable in part to difference in the parasite virulence. Malariatherapy using *P. vivax* to treat neurosyphilis highlighted differences in the parasite strains. Some strains induced infections curing without antimalarial treatment, whereas others caused severe infections and were associated with case-fatality rates of 10–14%.

5. *Plasmodium vivax* and the Duffy antigen in Africa

P. vivax is absent or rare in West and Central Africa and has a very low prevalence in East Africa. The gap in distribution of *P. vivax* in Africa compared to the rest of the world is viewed as the consequence of the lack of expression of the Duffy antigen on the red cells in the African populations in whom homozygosity for the null allele is highly predominant. In support of this view, although less frequent than *P. falciparum* in East Africa, *P. vivax* is endemic in some populations of Sudan, Somalia and Ethiopia who are predominantly Duffy positive. A study conducted in communities from Ethiopia at risk to malaria showed Duffy-positive rates ranging from 8% in the Nilotes to 70% in the Hamito-Semites. The relative prevalence of *P. vivax* mirrored the ratio of Duffy-positive in the communities (2.4% for the Nilotes and 27.3% for the Hamito-Semites) [31]. The exact frequency of *P. vivax* in East Africa however is unknown, and to be honest, the exact frequency of Duffy blood group is poorly documented across Africa, as indeed few populations have been surveyed and there are large gaps in our documentation on Duffy genotypes and phenotypes across Africa.

Of 50,000 specimens from West Africa that they examined, Escudie and Hamon did not find any evidence for the presence of *P. vivax*, although they observed the three other human malaria species [32]. Using a sensitive and species-specific PCR-based methodology, we did not find any evidence for the presence of *P. vivax* infection in a Senegalese setting where the other three species of malaria parasites are transmitted [33].

However, there seems to be some *P. vivax* circulation in these areas. The TropNetEurop network that recorded 585 imported *P. vivax* mono-infections in European countries from 1999 to 2003, reported that 5.5% of these were travellers infected in Central Africa. Surprisingly, for 11.4% of the *P. vivax* cases, the region of infection was Western Africa, more than the 10.6% of cases who were infected in Eastern Africa [34]. To look for the presence of *P. vivax* in Africa, a PCR-based survey was conducted in 2588 persons from nine African malaria endemic countries. This study found no evidence of *P. vivax* malaria in any of the persons, except one case, who was a Duffy-positive inhabitant from Sao Tome, where Duffy-positive and Duffy-negative populations live. The authors concluded that there are sufficient numbers of Duffy-positive individuals in some areas in Africa to maintain *P. vivax* transmission in areas where the majority of the population is Duffy-negative [35].

6. Malariatherapy and experimental inoculations

Malaria therapy used from the 1920s to 1960s to treat neurosyphilitic patients showed that African American patients were highly resistant to *P. vivax* blood-stage malaria [36]. Several strains of *P. vivax* were used for such therapies, including ''domestic'' strains from the United Sates (usually from South Carolina) and strains from other endemic regions. Data point to refractoriness of African–American patients to *P. vivax* inoculation, although there were exceptions.

Experimental infections of healthy subjects were also conducted to investigate the lack of susceptibility of African–American populations to *P. vivax*. Boyd and Stratman-Thomas infected ''southern negroes'' with *P. vivax* parasites of domestic origin by means of large numbers of infected mosquitoes [37]. Three of six adults failed to develop malaria, two developed brief fevers, and one developed a six-day fever twelve days after infection followed by one brief recurrence. Inoculation of control ''white'' volunteers with the same batches of infected mosquitoes invariably induced malaria. Other series of mosquito-bite inoculations induced malaria in the ''white'' recipients but consistently failed to induce infection in ''negroes'' even after they were exposed to many more infective bites than the ''whites'' [37]. Infections by the bite of infected mosquitoes or by injection of infected blood of *P. vivax* strains of different geographic origins, including the Chesson strain, in ''negroe'' and ''white'' subjects showed close to 100% infection in the ''white'' recipients, and only 23% in the ''negro'' people [36]. Young et al. discussed that these data confirmed similar findings for all strains of vivax tested, which originated from countries such as Tunisia, Sicily, Italy, Korea and the South-West Pacific, and concluded that African Americans have a ''general resistance to strains from all areas'' [38].

To investigate the hypothesis that rarity of *P. vivax* in Liberia was due to the insusceptibility of local populations to these parasites, Bray inoculated the Madagascar strain of *P. vivax* in 30 Liberians, who presumably were Duffy-negative. Only one of these (of unknown Duffy blood group) showed a patent blood-stage *P. vivax* infection, all the other being fully refractory to parasite inoculations (unlike a Duffy-positive recipient who readily became infected). Bray concluded: ''it is obvious that Liberians of all ages are highly resistant to infection with the Madagascar strain of *P. vivax*, as was to be expected from the results of previous work. This factor is obviously the main cause of the absence or rarity of *P. vivax* in Liberia'' [39].

After the discovery of the Duffy blood group, landmark in vivo studies by Miller et al. conclusively showed that Duffy-negative people resisted, while Duffy-positive people were susceptible to *P. vivax* blood-stage infection induced by exposure to infected mosquitoes [40]. Five different strains were used in this study, and infection was done by mosquito bites. Each infection experiment included Duffy-negative and Duffy-positive individuals, including Black Fy(a+b–) or Fy(a–b+) heterozygotes. None of the five Fy(a–b–) black subjects developed any patent blood stage infection, whereas all other

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volunteers were parasitaemic after a 9–15 days prepatent period. This definitively showed that the genetic resistance factor for vivax malaria was the Duffy blood group determinant and that homozygosity for the null allele conferred total refractoriness to infection to vivax malaria [40].

7. In vitro studies on *Plasmodium vivax* invasion

The demonstration that Duffy-negative homozygotes do not develop *P. vivax* blood stage infection was followed by in vitro experiments demonstrating that the Duffy blood group was the receptor for *P. vivax* [40]. This has become since a paradigmatic example of innate resistance to an infectious agent because of the absence of a receptor for the agent on target cells. Studies on the related *P. knowlesi* parasite, a species that infects macaques — and recently shown to cause zoonoses in humans [41] — were used as ''model'' for *P. vivax*, in particular with regard to invasion mechanisms [42].

Miller et al. studied invasion of *P. knowlesi* merozoites into human red cells of various Duffy phenotypes [42]. The invasion rates for the three Duffy positive phenotypes, Fy(a+b–), Fy(a–b+), and Fy(a+b+) were similar, but invasion was nil or negligible in Duffy-negatives (Fya–b–). Video recording showed that although *P. knowlesi* merozoites attached to Duffy positive as well as to Duffy-negative red cells, but the complete invasion process, which involves a subsequent localised invagination of the red cell round the merozoite, did not occur with Duffy-negative red cells. This indicated that – at least in the case of *P. knowlesi* – initial attachment to the red cell surface is Duffy-independent but that the following step, which involves the formation of a tight junction, is Duffy-dependent [42]. It has since been shown that this process involves a ''Duffy binding protein'' orthologous to the *P. vivax* Duffy binding protein (PvDBP, see below).

P. vivax invasion of human red cells was studied by Barnwell et al. using a short term in vitro assay and *P. vivax* parasites (Belem strain) obtained from squirrel (*Saimiri sciureus*) monkeys [43]. Human Duffy-positive Fy(a+b+) and human Duffy-negative Fy(a–b–) erythrocytes along with various simian erythrocytes were used as target cells for invasion. *P. vivax* did not invade Duffy-negative human erythrocytes, while Duffy-positive erythrocytes were infected. Infection was blocked by an anti-Fy6 mAb. *P. vivax* also invaded in vitro the erythrocytes of *Aotus* and *Saimiri* monkeys that lack Fya and/or Fyb Duffy determinants but carry the Fy6 and Fy3 determinants (*Saimiri* monkeys have an Asn to Ser substitution at codon 44 [44]). The Fy6-negative rhesus red cells were not invaded by *P. vivax* but were invaded by *P. knowlesi*, and *P. knowlesi* [43] possesses an alternative Duffy-independent invasion pathway.

8. Mechanism of RBC invasion of *Plasmodium vivax* merozoites

P. vivax infects almost exclusively reticulocytes. The molecular basis of this is still not fully elucidated, although it is clear that *P. vivax* Reticulocyte Binding Proteins (PvRBP) come into play. PvRBP is displayed on the surface of the invading merozoite and binds to an unknown receptor present on the surface of the reticulocyte. Sequencing showed that the *P. vivax* genome has ten *pvrbp* genes, the exact function of which is still unclear [45].

The Duffy antigen is recognised by a second ligand exposed unto the invading merozoite surface and named the PvDBP. PvDBP is encoded by a single copy gene in the *P. vivax* genome [45]. It is secreted from the micronemes of the *P. vivax* merozoite and discharged onto the surface of the merozoite at the time of invasion (reviewed in [46]). Its binding to the target red blood cell results in the formation of the tight junction that the parasite uses to penetrate into the host cell. The receptor-binding domain lies in cysteine-rich region II of the PvDBP (PvDBPII) [47].

The binding site for PvDBP region II maps to amino acids residues Ala8-Asp42 at the N-terminal extracellular region of the Duffy antigen. A 35 amino acid peptide from this region of Duffy blocks binding of Duffy-positive red cells to PvDBPII expressed on the surface of COS cells [48]. Using site directed mutagenesis of recombinant Duffy antigen, the PvDBPII binding region was delineated between residues Q19 and W26, which corresponds to the Fy6 determinant recognised by the BG6 mAb shown to inbibit invasion of human erythrocytes by *P. vivax* in vitro [43,49]. This is not to say that additional residues of the extracellular domain are not important. Indeed, sulfation of Tyr41 increases PvDBPII/Duffy antigen affinity by up to 1000-fold and can reasonably be considered essential for PvDBPII binding [50].

Consistent with an essential role for PvDBP in invasion, antibodies raised to recombinant PvDBPII inhibited binding of PvDBPII to the Duffy antigen, reacted by immunofluorescence with the merozoite and reduced the invasion of *P. vivax* parasites into Duffy-positive erythrocytes in vitro [51]. PvDBP is immunogenic in humans infected with *P. vivax* and apparently induces antibodies that contribute to protection. The presence of antibodies that inhibit the PvDBPII/Duffy interaction was negatively correlated with *P. vivax* infection [52]. In the field, PvDBP is polymorphic. Based on the crystal structure of the related *P. knowlesi* Duffy binding protein [53], the critical residues for receptor binding could be mapped to an area so-called sub-domain 2. Interestingly, the Duffy-recognition site and clusters of polymorphic residues lie on opposite sides. This spatial segregation probably reflects the ''last minute'' delivery of the ligand from the micronemes to the merozoite surface, such that binding to the receptor immediately follows exocytosis, leaving the residues exposed on the opposite face accessible for antibody binding... and immune selection.

9. *Plasmodium vivax* infections in Duffy-negative patients

Data published in the older literature report cases of *P. vivax* infections in people of African descent. In particular, a survey of Georgia school children conducted in 1943 showed that 57% of the parasitaemic ''white'' school children had a *P. vivax* infection compared to 18% of the parasitaemic ''negro'' school

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children (Bispham 1943, quoted by [54]). Frequent *P. vivax* malaria episodes were recorded in African American troops stationed in a highly malarious of the South Pacific (Melanesia) during the Second World War [54]. Such studies are difficult to interpret because *P. vivax* is morphologically difficult to distinguish from *P. ovale* and because the Duffy blood group of the patients have not been determined. The possibility exists therefore that the reported *P. vivax* cases were actually *P. ovale* infections and/or that the black patients were not homozygous for the null allele and were not Duffy-negative. A study conducted in Honduras reported that 19% of persons characterised as ''white'' were Duffy negative by serologic typing, reflecting significant black admixture in the population [55].

However, a number of recent reports concern findings of *P. vivax* in the blood of Duffy-negative persons. First, in western Kenya, Ryan et al. reported blood infection by parasites presenting the morphological characteristics of *P. vivax* in nine children who were phenotyped as Duffy negatives by flow cytometry for the Fy3 or Fy6 determinants [56]. *P. vivax* parasite densities were low; all children were also infected with *P. falciparum* and some were also infected with *P. ovale* or *P. malariae*. However, species assignment could not be confirmed for some of the slides upon subsequent blinded examination by experienced microscopists. A DNA fragment from *P. vivax* merozoite surface protein 1 was amplified from the blood of four infected children, providing confirmation of infection by the *P. vivax* species. Low densities and mixed infections complicated the analysis, but there was at least one Duffy negative child with microscopic evidence for *P. vivax* blood stage infection that was confirmed by PCR. A small fraction of Anopheles mosquitoes from the area were positive for *P. vivax* circumsporozoite protein by Elisa, and PCR-positive for the *P. vivax*-SSU rRNA gene. Altogether these data indicate that *P. vivax* is transmitted in the region and capable of infecting some Duffy-negative individuals [56].

Subsequently, Cavasini et al. identied two cases of *P. vivax* in Duffy-negative individuals living in the Brazilian Amazon [57]. *P. vivax* parasites were detected using a PCR-based methodology in two phenotypically and genotypically Duffy negatives. The *P. vivax* primers used targeted the circumsporozoite locus and identified the VK210 and/or *P. vivax*-like variants, indicating that at least two different ''strains'' were able to infect Duffy-negative individuals [57]. A study of 312 vivax malaria patients with microscopically positive blood smears or positive PCR recruited in four areas from Brazil found two Duffy negatives patients with evidence of *P. vivax* [58]. It is unclear whether for these two patients, erythrocytes infected by *P. vivax* have been observed.

10. Assault to the paradigm in Madagascar

Compelling evidence that *P. vivax* is able to infect Duffy-negative red cells was obtained recently in Madagascar [15]. Human settlement in Madagascar, which is 250 miles off the east African coast, is recent. The island has been peopled by a succession of Austronesian and African migrants and more recently Europeans and Asians over the past 2500 years. The human populations display a full range of Duffy erythrocyte expression phenotypes, while the four main malaria parasites affecting humans are endemic. We conducted a study where we investigated the association of Duffy blood groups and susceptibility to infection by *P. vivax* among asymptomatic children and malaria patients. Study subjects were recruited in eight sites serving as sentinel sites for the surveillance of antimalarial drug susceptibility. Duffy locus genotyping of 661 asymptomatic children showed that a large proportion carried the $FY*B^{ES}$ allele (allele frequency = 0.83) and 72% were homozygous $FY*B^{ES/}B^{ES}$. PCR species diagnosis based on the small subunit (SSU) rDNA assay indicated that 42 of the 472 Duffy-negative samples had *P. vivax* parasites in the blood (8.8% *P. vivax* prevalence). Many (76%) of these *P. vivax*-positive Duffy negative children had *P. vivax* pure infections. All 42 infections were confirmed by a second *Plasmodium* species PCR assay based on cytochrome oxidase 1 (CO1). This was a first indication that *P. vivax* did infect Duffy negatives and showed that it was a rather frequent occurrence.

We then studied 183 *P. vivax*-infected samples from patients seeking malaria treatment from six different health facilities in Madagascar. *P. vivax* mono-infections were found in 153 patients and *P. vivax/P. falciparum* mixed infections were detected in 30 patients. In these patients, the frequency of the allele $FY*B^{ES}$ was 0.44. There were 17 homozygous $FY*B^{ES}/B^{ES}$ *P. vivax* patients, including nine in whom the various PCR assays identified only *P. vivax* and no other *Plasmodium* species. Intraerythrocytic parasites were detected on blood smears. Parasite densities were around 3000 intraerythrocytic forms per microliter. To reach such densities, *P. vivax* must have invaded and fully developed through multiple cycles.

To ascertain that the Duffy phenotype matched the genotype, red blood cells were typed using conventional serology, flow cytometry, and adsorption-elution methods. All three methods indicated a perfect match between genotypes and phenotypes. In particular, flow cytometry showed a gene-dosage dependent pattern and was fully concordant with the genotypes. Thus in this genetic environment persons with a Duffy negative genotype had indeed no Duffy antigen on the surface of the red cells. The conclusion of both studies thus was that in Madagascar, *P. vivax* is able to infect and cause disease in Duffy negative individuals. This capacity is not restricted to a single strain. In contrast multilocus genotyping using microsatellite, drug resistance and surface antigen genes showed that multiple *P. vivax* strains could infect Duffy negative people.

Duffy-negative patients with *P. vivax* malaria were observed at five of six study sites and we detected *P. vivax*-infected Duffy-negative asymptomatic children in four of eight study sites. Interestingly the prevalence of *P. vivax* infections in Duffy-negatives was related to their relative proportion in the local population. In the populations with a minority of Duffy positives, *P. vivax* infections were confined to Duffy positives and no *P. vivax* infection was detected in Duffy negatives. In contrast, in the sites where the ratio of Duffy-positive vs. Duffy-negative inhabitants was the highest, there was a high rate of infection in *P. vivax* Duffy negatives. This is reminiscent of

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observations by Mathews and Armstrong in Ethiopia [31]. The geographic heterogeneity in Madagascar needs to be further explored in future studies, as well as the population structure of the parasite. Nevertheless, it seems that sympatry of Duffy-positives and Duffy negatives associated with a relatively high prevalence of infections provides the conditions for *P. vivax* parasites to infect Duffy negatives. *P. vivax* gametocytes were observed in Duffy negative, indicative of the ability to transmit to mosquitoes. However, proof of the transmission from Duffy negatives has not been obtained and will need specific investigations. The observation that in areas such as Tsiroanomandidy nearly half the vivax infections were in Duffy negative individuals, who accounted for 52% of the population surveyed is a matter of concern. It suggests that indeed *P. vivax* has broken through its dependence on the Duffy antigen for establishing blood stage infection in Duffy negatives.

The mechanisms involved in the invasion of *P. vivax* in Duffy-negative erythrocytes remain to be elucidated. Clearly, these parasites use alternate pathways for entry into red cells. This has not been described yet for the *P. vivax* species. There are multiple entry pathways for *P. knowlesi* and importantly for *P. falciparum*. There are two main possibilities to account for the observations in Madagascar: either the parasites have acquired this possibility locally, i.e. in the specific geographical and ecological niche of Madagascar where Duffy negative and Duffy positive people coexist, or the specific situation of Madagascar with relatively high prevalence of vivax in the malaria transmission areas has allowed to unveil mechanisms that remain cryptic in most conditions. It is crucial to elucidate the molecular mechanisms involved in order to adapt the vaccination strategies accordingly, in particular the vaccines targeting the PvDBP that are currently under development.

11. Conclusions

Until now, the Duffy negative phenotype was viewed as giving almost total protection against infection with *P. vivax*. The mechanism of this protection is that Fy(a–b–) prevents *P. vivax* from invading host erythrocytes and completing its complex life cycle. The survival benefit in malaria-endemic regions of Africa may have provided the selective pressure to drive the allele to fixation in Africa, although phylogenetic analysis rather suggests that vivax was a zoonotic infection acquired by man in Asia [59,60]. Furthermore, it must be noted that other red blood cell polymorphisms that are nowadays highly prevalent protect against vivax as well and may interact with Duffy in certain populations [61]. Whatever the history that led to the present day situation, in vast regions of Africa inhabited by Fy(a–b–) human populations *P. vivax* is nowadays quite rare. The evidence obtained in Madagascar that multiple *P. vivax* strains are capable of bypassing the Duffy-negative invasion hurdle suggests recent evolution of the parasite. *P. vivax* has gained the capacity to cause human disease. Key questions for the future will be to investigate the transmission potential of these strains and prevent them from spreading away from Madagascar and to investigate whether similar parasite evolution occurs and possibly in other regions of the world where there is significant admixture of Duffy-positive and negative populations.

Conflict of interest

None.

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